





Article

The Effect of Increased Ozone Levels on the Stable Carbon and Nitrogen Isotopic Signature of Wheat Cultivars and Landraces

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Abstract: Several studies have highlighted the negative effects of ozone (O_3) on wheat development and productivity. The negative effects of O_3 are mediated by changes in photosynthetic carbon and nitrogen metabolism, which are difficult and time-consuming to assess and are thus only measured sporadically throughout the plant cycle. Stable isotope measurements in grains can help integrate the effects of chronic O_3 exposure over the lifespan of the plant. This particular study focuses on the extent to which the stomatal conductance and productivity of Mediterranean wheat are related to carbon and nitrogen isotopic signatures under chronic O_3 exposure. An open top chamber experiment was designed to analyse the effects of the pollutant on 12 Spanish wheat genotypes, which included modern cultivars, old cultivars and landraces. Four O_3 treatments were considered. Stomatal conductance (g_s) measurements were carried out during anthesis, and yield and nitrogen content parameters were taken at maturity, along with the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic composition measured in grains. Modern and old cultivars responded similarly to O_3 and were sensitive to the pollutant regarding yield parameters and g_s , while landraces were more O_3 -tolerant. Grain $\delta^{13}C$ had a strong negative correlation with grain yield and stomatal conductance across genotypes and O_3 conditions, and increased under higher O_3 concentrations, showing its capacity to integrate O_3 stress throughout the wheat cycle. Meanwhile, a higher nitrogen concentration in grains, coupled with smaller grains, led to an overall decreased grain nitrogen yield under higher O_3 concentrations. This nitrogen concentration effect within the grain differed among genotypes bred at different ages, following their respective O_3 -sensitivity. $\delta^{15}N$ showed a possible indirect effect of O_3 on nitrogen redistribution, particularly under the highest O_3 concentration. The correlations of $\delta^{15}N$ and $\delta^{13}C$ to the usual effects of ozone on the plant suggest their potential as indicators of chronic ozone exposure.

Keywords: isotopes; Mediterranean; genotypes; sensitivity; tolerance; breeding



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1. Introduction

Even though tropospheric ozone (O_3) is both a naturally occurring and anthropogenic gas, current global levels are mainly caused by photochemical reactions, due to precursors from industrial and urban emissions [1,2]. Local and regional air masses can transport precursors throughout long distances before leading to ozone production. When high ozone precursors and O_3 -favorable meteorological conditions for atmospheric chemistry coincide, pollutant formation increases, causing chronic and extensive O_3 -pollution problems in rural and forested areas, far away from the source of emissions [3]. This, along with the complexity of atmospheric O_3 photochemistry, coupled with high NO_x levels, explains the difficulty in maintaining O_3 levels under safe values for crops and ecosystems [2,4]. In Europe, several EU Directives have prompted the reduction of O_3 -precursor emissions

(2008/50/EU and 2016/2284/EU), which caused the high O₃ peaks that were of maximum concern during the eighties. Nonetheless, background O₃ levels still do not present a clear decreasing trend [5,6], and safe levels are not expected to be reached by the year 2030 [2].

Regarding chronically-high O₃ levels, the Mediterranean basin is the most affected area in Europe [5,7]. Its stable summer conditions, high temperatures and high solar radiation favour photochemical O₃ formation, which causes surface levels during spring and summer that surpass the threshold targets of the EU Directives (2008/50/EU and 2016/2284/EU) and the critical levels of the United Nations Air Convention for plant protection [5,8,9].

Along with the pollution problem, the expected impact of Climate Change is also recognized as a major concern in the Mediterranean area, a transition zone located between the arid North African climate and the temperate central European climate, which is prone to suffer substantial climatic changes [10,11]. Temperature is expected to increase by up to 5 °C by 2100 and, coupled with 25% less precipitation in the Mediterranean basin, these changes are expected to greatly harm crop yields [11] and worsen the O₃ pollution problem [6,12].

O₃ affects the plant by entering through the leaf stomata and producing reactive oxygen species (ROS), which react with the cell membranes and walls [13]. This early O₃-induced oxidative burst drives cellular chain effects that alter physiological processes such as carbon assimilation by affecting stomatal conductance (g_s), inducing partial closure or less efficient stomatal control [14], reducing foliar pigment content [15], affecting the electron transport chain [16], reducing the efficiency of Calvin cycle enzymes such as Rubisco [17], reducing photosynthates made available for redistribution [18] and increasing respiration rates [19]. As a final result, yield and grain quality are reduced [20,21]. Over 20% of all crop production in Europe is currently considered at risk of damage by O₃ pollution, as O₃-sensitive and moderately sensitive crops are grown in around 16% and 7%, respectively, of the agricultural land in Europe [21,22].

Wheat, one of the most important staple crops on a global scale, is also one of the most O₃-sensitive crops [21,23,24]. Studies from the last decades have shown that this pollutant can induce important wheat yield losses and reduce grain quality (i.e., starch content, seed size), while also affecting N use efficiency and protein yield [25]. O₃-induced wheat yield reduction is important both for its economic impact and its effect on global food security. In terms of wheat yield, modelling exercises have shown that O₃ pollution can account for an average global reduction of 7.1% [26], ranging between 4.1–12.1% for Europe [19]. Moreover, data worsens when coupled with other constraints such as climate change and pressures to maintain natural resources [27].

Breeding for higher grain yield (GY) in wheat and other herbaceous crops has been coupled frequently with an enhancement of stomatal conductance (g_s), which favours higher photosynthetic rates and cools down plants [28–31]. However, the higher g_s of modern wheat cultivars can also potentially increase their O₃-sensitivity, as they absorb the pollutant more readily. This has led to a clear trend in the genotypic variation regarding how wheat cultivars respond to O₃. In general, modern cultivars are more O₃-sensitive than older ones [28,32]. This also applies to wheat bred for Mediterranean conditions, although their adaptation to water-limited environments might have also made them more O₃-tolerant (Bermejo-Bermejo et al., in press).

Ozone-induced effects in plants are usually studied through discrete gas exchange measurements during the crop season [28,33,34]. However, as these measures are very time consuming, they are usually only performed a small number of times throughout the growth cycle [26,35,36]. Usually, what is observed in these instantaneous measurements is extrapolated to the entire cycle, even though O₃ responses can differ depending on the plant growth stage, with grain filling appearing to be the most susceptible stage [37,38]. A better-integrated response of plant gas exchange and assimilation can be analysed through the study of the stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope compositions [39–42], particularly when analysed in grain after harvest.

Carbon fractionation is driven by the discrimination of stable carbon isotopes, favouring the uptake and further assimilation from the surrounding air of the lighter isotope, ^{12}C , against ^{13}C [39,43]. It should, however, be noted that frequently, rather than expressing the isotope signature as a composition (denoted as δ) which is the deviation of the $^{13}\text{C}/^{12}\text{C}$ molar abundance ratio between the analysed substance and a standard, values are transformed to discrimination (denoted as $\Delta^{13}\text{C}$) of the ^{13}C from the atmosphere surrounding the plants [43]. From a physiological point of view, this discrimination depends on the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) [39,43]. If this ratio is integrated over time, it shows the balance between the g_s and the net photosynthetic assimilation of the plant during the considered period [43–45]. Thus, $\delta^{13}\text{C}$ discrimination occurs due to the diffusion of CO_2 through the stomata and by Rubisco carboxylation [43,46]; therefore, if the stomata close, for example as a response to water stress, g_s decreases, restricting the CO_2 supply to the carboxylation sites, and overall decreasing the C_i/C_a and consequently $\Delta^{13}\text{C}$, while increasing $\delta^{13}\text{C}$ [39,40,43].

Meanwhile, $\delta^{15}\text{N}$ values are driven by discrimination of ^{14}N against ^{15}N , which depends largely on the capacity of the plant to assimilate the nitrogen available in the soil. Thus, $\delta^{15}\text{N}$ in the plant may reflect $\delta^{15}\text{N}$ of the nitrogen available in the soil, as well as metabolism, including recycling, transport through the plant, including the grains and even volatilization during senescence [38,47,48].

In that sense, changes in g_s may affect the transpiration stream and, subsequently, nitrogen uptake from the soil, its transport to the leaves and volatilization, thus affecting $\delta^{15}\text{N}$ [49]. Similarly, retranslocation of nitrogen through the plant via phloem, as well as photorespiration, can cause $\delta^{15}\text{N}$ to increase in the upper organs, whereas roots tend to have lower $\delta^{15}\text{N}$ than the stems and leaves, and these have less $\delta^{15}\text{N}$ than the ears [47]. Nitrogen uptake after anthesis is quite low [50], therefore, as O_3 -effect is more predominant post-anthesis [37], N redistribution could be expected to be affected more than uptake by the pollutant.

In the present study, the effects of O_3 exposure on the stable carbon and nitrogen isotope composition and its relation to yield parameters, as well as g_s , were studied, considering a set of Mediterranean bread wheat genotypes that included landraces, along with old and modern commercial varieties, of which all except for one were developed after the so-called Green Revolution, which took place in the middle of the last century. The objective of the study was to assess the performance of grain $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as an integrated tool to monitor chronic O_3 effects on C assimilation and N metabolism in Mediterranean wheat genotypes. Both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ have been shown to perform as useful indicators in wheat under other types of stresses, such as water-stress and salinity [39,42,51].

The starting hypotheses were that higher O_3 -stress will decrease g_s , increasing $\delta^{13}\text{C}$ (or lowering carbon isotope discrimination, $\Delta^{13}\text{C}$) particularly on the more O_3 -sensitive genotypes, in comparison to the tolerant genotypes. We postulate that this would cause a negative correlation between $\delta^{13}\text{C}$ and yield parameters or g_s due to the effect of O_3 , differing among wheat genotypes developed at different times. Even though the mechanisms explaining $\delta^{15}\text{N}$ are less established than those of $\delta^{13}\text{C}$, since N absorption, metabolism and redistribution affect the nitrogen isotope signature, continuous O_3 -exposure is also expected to affect $\delta^{15}\text{N}$ of mature grains.

2. Materials and Methods

2.1. Field Site

The experiment was developed in the CIEMAT Open Top Chambers (OTCs) facility located at “La Higuera” MNCN/CSIC Research Farm (Santa Olalla, Toledo) in central Spain (450 m.a.s.l.; 40°3′ N, 4°26′ W) (Figure 1), which was specifically designed to study the effects of increasing O_3 levels on crop plants. According to the Köppen–Geiger climate classification, this area experiences a hot-summer Mediterranean Climate (Csa) [52] and is used for rainfed cereals, characteristic of the agriculture of the central Iberian Peninsula.



Figure 1. Location of the CIEMAT OTC facility at the “La Higuera” MNCN/CSIC Research Farm (Santa Olalla municipality, Toledo, Spain).

2.2. Open Top Chamber Facility

The experiment employed fifteen NCLAN-type OTCs, which are circular-like greenhouses (3 m high \times 3 m diameter) that are open at the top through a conic structure (*frustum*) to prevent wind from entering the chambers [53].

The experimental design of the facility included a complete randomized 3-block design with four O₃ treatments replicated three times: thus, 3 OTCs per O₃ treatment: Charcoal Filtered Air (FA), Non-filtered air (NFA) to reproduce ambient levels, Non-filtered air + 20 nL L^{−1} of O₃ (NFA+) and Non-filtered air + 40 nL L^{−1} of O₃ (NFA++); where the last two reproduce O₃ levels that are expected along this decade, as well as three chamberless plots (ambient air—AA) to check for chamber effect. More detailed information on the OTC facility can be found in Calvete-Sogo et al., 2016 [54].

2.3. Instrumentation OTC Facility

An O₃ generator (A2Z Ozone Systems Inc., Louisville, KY, USA) system, located in a control cabin and fed with pure O₂, was used to apply O₃ to the NFA+ and NFA++ OTCs for 8 h day^{−1} (6:00 to 14:00 GTM), seven days a week. Air pollution was monitored continuously above the canopy inside each of the OTCs and AA plots by employing a timesharing system, with a sampling period of 10 min per plot. The air pollutants measured were: Ozone (ML[®] 9810B, Teledyne Monitor Labs, Englewood, CO, USA) and nitrogen oxides (NO₂ and NO; ML[®]9841, Teledyne Monitor Labs, Englewood, CO, USA). Monitors were calibrated at the beginning of the experiment following a standard protocol.

Meteorological parameters were also monitored within six of the OTCs and the three AA plots: air relative humidity (RH), temperature (HOBO[®] Pro v2, Onset, Bourne, MA, USA) and photosynthetic active radiation (PAR; OSO-SUN HOBO[®], Onset, Bourne, MA, USA).

2.4. Ozone Exposure Indices

Several ozone exposure indices were calculated to describe ozone exposure during the fumigation period: the accumulated AOT40 index was calculated as the sum of exceedances of O₃-hourly mean values over 40 nL L^{−1} during daylight hours through the O₃-exposure

period [55]. In addition, 24 h-mean, 7 h mean and maximum hourly O₃ values were also calculated.

2.5. Plant Material

Twelve Spanish wheat genotypes were selected for the assay: 4 modern commercial varieties (CVs), 4 old CVs and 4 landraces (Table 1). Pane 247 and the landraces were released pre-green revolution. The Plant Breeding Unit of the Agronomic Engineer School of Madrid (UPM) provided the seeds which were germinated in a plant nursery greenhouse. Seedlings were transported to the OTC field for transplanting into pots (18 cm³) using a mixture of turf (60%), perlite (20%) and vermiculite (20%) on January 26–28 of the year 2015. Four plants per pot were considered as well as four pots per cultivar and OTC (totalling 12 pots per CVs and O₃ treatment). Pots were fertilized following the commercial management of wheat fields in the area, with 120 kg N ha⁻¹ applied in two doses, on April 14th and 30th. Regarding irrigation, plants were watered manually based on the plant needs at the time (e.g., climatic conditions) to maintain water saturation at field capacity, approximately every two days, until the beginning of grain hardening towards the end of May, at which point watering stopped to allow for grain maturity.

Table 1. Wheat genotype grown under Mediterranean conditions, Genotype Age, Harvest Date, Growth Habit, Year of Release and References. The dashed line represents pre and post green revolution release dates.

Genotype Age	Genotype	Harvest Date	Growth Habit	Year of Release	References
Modern	Nogal	June 9	Winter	2006	Grupo para la Evaluación de Nuevas Variedades de Cultivos Extensivos en España (GENVCE) database ^a
Modern	Arthur Nick	June 3	Spring	2002	
Modern	Berdún	June 10	Winter	1998	
Modern	Califa Sur	June 3	Spring	1999	
Old	Marius *	June 10	Winter	1976	Genetic Resources Information and Analytical System (GRIS) database ^b
Old	Yécora	June 9	Spring	1972	
Old	Ablaca	June 18	Spring	1982	
Old	Pane 247	June 17	Spring	1955	
Landrace	Aragón	June 18	Winter	<1940	Spanish National Plant Genetic Resources Centre (CRF) database ^c
Landrace	Chamorro	June 23	Winter	<1940	
Landrace	Mocho Rojo	June 17	Spring	<1940	
Landrace	Candeal de Vellisca	June 23	Spring	<1950	

^a GENVCE. Fichas archivos. 2021. <https://genvce.org/productos/fichas/> (accessed on 4 July 2021). ^b GRIS. Genetic Resources Information System for Wheat and Triticale. 2021. <http://wheatpedigree.net> (accessed on 4 July 2021). ^c CRF. National Plant Genetic Resources Centre (CRF) Databases. 2021. <http://wwwsp.inia.es/en-us/Investigacion/centros/crf/databases/Paginas/inicioDatabases.aspx> (accessed on 4 July 2021). * Marius is a reference CV for Central Spain.

Plant O₃ exposure began on April 14th, 77 days after transplant to pot (DaT), when plants were close to the anthesis stage. Fumigation lasted for 51–71 days, depending on the CV, until plants reached full grain maturity and were harvested.

2.6. Physiological and Yield Parameters

Stomatal conductance (g_s) measurements were performed using a portable gas exchange system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA) between 9:00–13:00 h GMT from May 4–14 (depending on the cultivar) after 20–30 days of exposure to the different O₃-treatments and, therefore, around 100 DaT. Two plants per OTC for two blocks were randomly selected for the measurements, with one flag leaf per plant. The standardized conditions during gas exchange measurements for all the cultivars were: PAR 1000 µmol m⁻² s⁻¹, RH 55% and a temperature range of 20–22 °C.

Yield, agronomical yield components and nitrogen content parameters were taken at grain maturity from June 3 to June 23 depending on each genotype (Table 1). After harvesting plants from the base, they were divided into straw and spikes, dried at 60 °C for 48 h and weighted to obtain the dry weight of the different plant parts (DW pot⁻¹), totalling 48 pots per CV and 12 pots per CV and O₃ treatment. Spikes were manually shelled to calculate the grain yield. Harvest Index (HI) was calculated as the ratio between

grain and the total weight of the aerial plant (straw plus grain husks). Individual grain weight was then calculated and further grain nitrogen content analysed as described below. The number of grains per ear was calculated by dividing the total number of grains over the total number of ears for each pot.

2.7. Nitrogen Content and Stable Carbon and Nitrogen Composition

After measuring harvest parameters, the whole grain production from each genotype and OTC was pooled for isotopic analysis (15 samples per genotype and 3 samples per O₃ treatment and genotype). Pooled grain per OTC and genotype was ground and analysed for total nitrogen content and ¹³C/¹²C and ¹⁵N/¹⁴N signature using an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan, Bremen, Germany) paired with an elemental analyser (Flash 1112 EA, ThermoFinnigan, Bremen, Germany). One-mg samples of the ground wheat, and reference materials, were weighed, placed in tin capsules, sealed and loaded onto an automatic sampler (ThermoFinnigan, Bremen, Germany) for EA-IRMS analysis. These measurements were carried out at the CCiT (Centres Científics i Tecnològics) at the University of Barcelona. Total nitrogen content was expressed as percentage (%) of dry matter, while the isotope ¹³C/¹²C ratio was expressed in δ notation [46]:

$$\delta^{13}\text{C} (\text{‰}) = (^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}} - 1$$

where “sample” refers to plant material and “standard” to the international secondary standards of known ¹³C/¹²C ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose, and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB). Analyses were performed with an analytical precision (SD) of 0.10‰. The ¹⁵N/¹⁴N ratio is also expressed in δ notation (δ¹⁵N); however, using international secondary standards of known ¹⁵N/¹⁴N ratios (IAEA N1 and IAEA N2 ammonium sulphate and IAEA NO3 potassium nitrate) calibrated against N₂ in the air, with an analytical precision of 0.18‰. Nitrogen as a concentration (N%) was converted into Nitrogen Grain Yield (NGY) following the function: NGY = (N% × Grain Yield)/100.

2.8. Statistical Analysis

Effects of O₃ and genotype age-type factors on the different variables were analysed via two-way analysis of variance (ANOVA) with IBM® SPSS® Statistics 20 (Chicago, IL, USA) using block as a random factor. Significant differences among levels of the factors ($p < 0.05$) were assessed with the Tukey Honestly Significant Difference test (HSD). The two-way ANOVA data were previously checked to fulfil the ANOVA requirements of normality and homoscedasticity through Shapiro–Wilk and Levene’s tests, respectively, using the SPSS Explore Descriptive Statistics function, with Plots. The same procedure was then followed with a one-way ANOVA analysis of each genotype, using the post-hoc Tukey’s HSD test. The old CV Pane 247 was excluded when the grouped analysis of the modern and old CVs was done, due to its proximity to the landrace group and its behaviour as an outlier within these age-groups.

Pearson correlations between δ¹³C and δ¹⁵N and stomatal conductance and yield parameters were analysed. The significance of the regressions was assessed by p -value ($p < 0.05$) and the goodness of fit was judged by the determination coefficient (R^2).

Finally, a Principal Component Analysis, without rotation and with Keiser Normalization, was performed. The standardized data, excluding Pane 247, were checked for Kaiser–Meyer–Olkin Measure of Sampling Adequacy and Bartlett’s Test of Sphericity for the usefulness of the factor analysis. Then, the components with an Eigenvalue higher than 1 were selected (leading to two components) and verified with the scree plot to check for a clean drop in the Percentage of Variance after PC2. Variables selected for each component presented a Component Matrix value higher than 0.5. This was repeated for the data in general, and then the data were divided by genotype group. The PCA images were acquired with R, version 3.6.3 (29 February 2020) to include the ellipses.

Table 2 summarizes the methodology and instrumentation for each experimental stage.

Table 2. Summary of the experimental conditions and instrumentation used in the experiment per stage.

Experimental Stage	Methodology	Instrumentation
Stage 1. Planting	January 26–28th: Seedlings transplanted into pots in the open top chambers (OTC)—four plants per pot—with four pots per CV and OTC, totalling 12 pots per CVs and O ₃ treatment. April 14th and 30th: Pots fertilized with 120 kg N ha ^{−1} in two doses Manually irrigated to maintain water saturation at field capacity (approx. every 2 days) until grain hardening in May.	12 cultivars—See Table 3 (3 Modern, 3 Old, 3 Landraces) 17,671.5 cm ³ pots Mix of turf (60%), perlite (20%) and vermiculite (20%)
	Randomized design with 3 chamberless plots and four O ₃ treatments in 3 blocks: <ul style="list-style-type: none"> • AA: Ambient Air (chamberless plots) • FA: Charcoal Filtered Air • NFA: Non-filtered Air • NFA+: Non-filtered Air + 20 nL L^{−1} • NFA++: Non-filtered Air + 40 nL L^{−1} 	Fifteen 3 m × 3 m NCLAN-type OTC with a frustrum.
Stage 2. Ozone Fumigation	O ₃ application to the NFA+ and NFA++ OTCs for 8 h day ^{−1} (6:00 to 14:00 GTM), 7 days a week. April 14th: fumigation begins 77 days after transplantation, lasting 51–71 days (depending on the CV) until plants reached full grain maturity.	A2Z Ozone Systems Inc., Louisville, KY, USA O ₃ generator system, located in a control cabin and fed with pure O ₂ , with system monitors calibrated at the beginning of the experiment.
	Monitoring of ozone and nitrogen oxide concentrations inside every OTC and AA plot (above canopy) every 10 min.	Ozone monitoring (ML [®] 9810B, Teledyne Monitor Labs, Englewood, CO, USA) Nitrogen oxides monitoring (NO ₂ and NO; ML [®] 9841, Teledyne Monitor Labs, Englewood, CO, USA).
	Monitoring of the air relative humidity (RH), temperature and photosynthetic active radiation (PAR) in 6 of the OTCs and all 3 AA plots.	RH and temperature monitoring: HOBO [®] Pro v2, Onset, Bourne, MA, USA. PAR monitoring: OSO-SUN HOBO [®] , Onset, Bourne, MA, USA.
	May 4–14th: Stomatal conductance (g _s) measurements taken between 9:00–13:00 h GMT after 20–30 days (depending on the cultivar) of O ₃ exposure. Two plants randomly selected per OTC for 2 blocks, measured on the flag leaf.	Li-Cor 6400; Li-Cor, Lincoln, NE, USA, with the standardized conditions: PAR 1000 μmol m ² s ^{−1} RH 55% Temperature range 20–22 °C
	June 3rd: Yield, agronomical yield components, taken after grain maturity and harvest <ul style="list-style-type: none"> • Dry weight of straw, spikes and grains • Number of grains • Spikes shelled manually to calculate GY. • HI, individual grain weight and number of grains per ear calculated per pot. 	Harvested plants divided into straw and spikes, which were later shelled, dried at 60 °C for 48 h and weighed.
Stage 3. Measurements	June 3rd: Yield, agronomical yield components, taken after grain maturity and harvest	
	Nitrogen content parameters and isotopic signalling (15 samples per genotype and 3 samples per O ₃ treatment and genotype)	EA-IRMS analysis: Elemental analyser (EA): Flash 1112 EA, ThermoFinnigan, Bremen, Germany. Ratio mass spectrometer: Delta C IRMS, Thermo Finnigan, Bremen, Germany.
	Analysis of variance (ANOVA) with Tukey Honestly Significant Difference test (HSD) Pearson correlations between δ ¹³ C and δ ¹⁵ N and g _s with yield parameters	IBM [®] SPSS [®] Statistics 20 (Chicago, IL, USA)
Stage 4. Statistical Analysis	Principal Component Analysis without rotation and with Keiser Normalization	R, version 3.6.3, factoMineR package *

* For further information, refer to: Lê, S., Josse, J. & Husson, F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Softw.* **2008**, 25.1, 1–18.

3. Results

3.1. Climatic Variables and Ozone Exposure

Figure 2a shows the mean daily O_3 profiles for the different O_3 treatments during the months of the experiment. Hourly mean FA values ranged between 20–35 $nL L^{-1}$, well reproducing the natural background levels currently found in the area during the winter and early fall seasons. NFA represents the common chronic high spring-summer levels of rural areas in the Central Iberian Peninsula, with values at the central hours of the day ranging between 50–60 $nL L^{-1}$. Fumigated treatments (NFA+ and NFA++) increased the maximum hourly values to around 65 and 90 $nL L^{-1}$, respectively. NFA values increased from April to June, following the O_3 -seasonal variability in the area, while the NFA+ and NFA++ values in June decreased and even matched NFA, following the fumigation shutdown on June 8th, when the plants were dry and the grain mature. April average values for the fumigated treatments were also lower due to the start of the fumigation on April 14th; before this date plants were grown under O_3 -ambient levels.

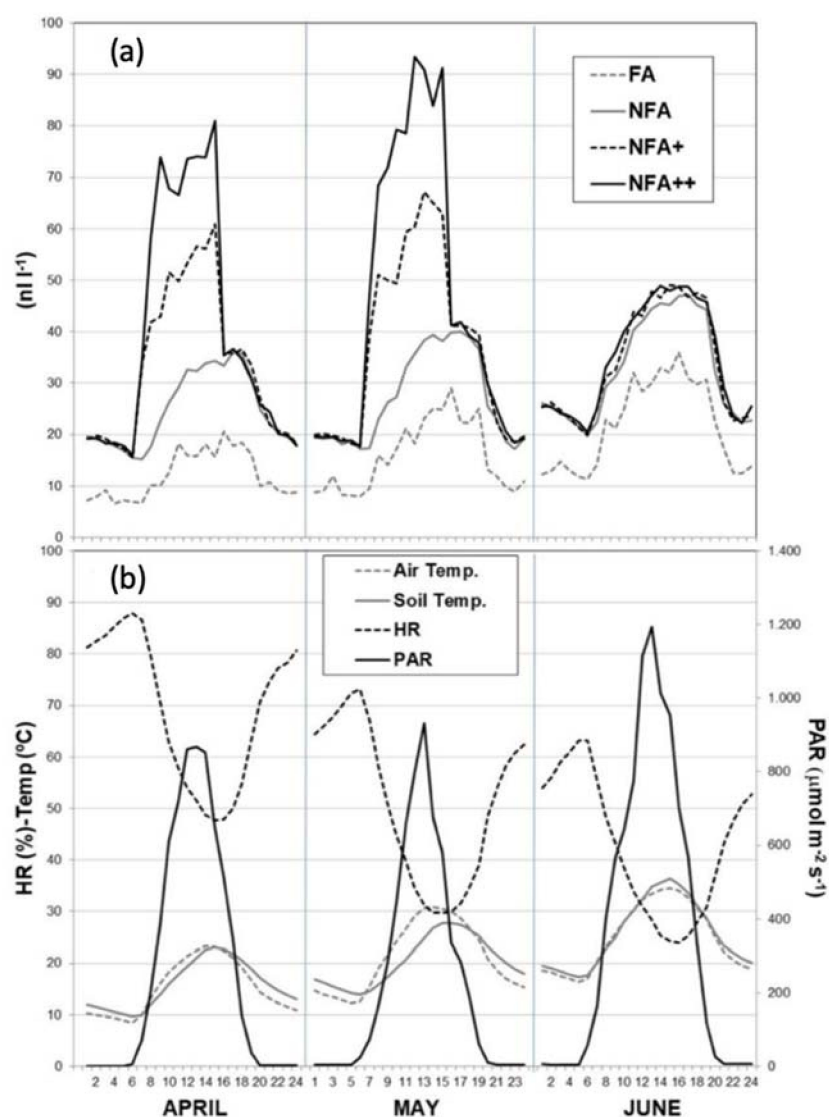


Figure 2. (a) Mean daily profiles of O_3 concentration for the different O_3 -treatments during the experimental period: FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 $nL L^{-1}$ of O_3 , NFA++ = non filtered air supplemented with 40 $nL L^{-1}$ of O_3 ; (b) Mean daily profiles of meteorological parameters during the exposure period inside the OTCs: HR (%), air and soil temperature (°C) and photosynthetic active radiation (PAR $\mu mol m^{-2} s^{-1}$).

Mean values for the meteorological parameters inside the chambers during the exposure period are represented in Figure 2b. All the parameters followed the seasonal trend of the climatic area at the OTC site. Air and soil temperature increased from April to June, with maximum mean daily values ranging from 22–36 °C for air, as the growth cycle progressed, and 22–34 °C for soil. On the contrary, RH values dropped from 48% to 24% between April and June, reaching the minimum mean daily values at midday (minimum hourly mean values around 24% in June). Photosynthetic active radiation (PAR) also increased throughout the cycle, ranging between 850–1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ inside the OTCs, and peaking in June at the end of the experiment.

3.2. Genotype Age-Type Effects

The age-type genotype factor caused clear significant differences in the means of all the parameters assayed: GY and GNY, g_s and stable isotopic composition (Table 3, Age-type factor). In general, no significant differences existed between the modern and old CVs, which, in turn, differed from the landraces significantly. More specifically, breeding caused an improvement in the GY of both categories of CVs, which increased by 34% (mean across O_3 treatments and cultivars) when compared to the landraces. Regarding HI, it increased by 37% in CVs compared to the landraces. Cultivars exhibited a higher number of grains per ear (by 54%) than the landraces, but at the expense of lower grain size. Thus, single seed size was 14% lower in CVs than landraces; also, breeding caused a decreased straw yield (SY) (38%) and total aboveground biomass (3%) (Appendix A: Table A1).

Table 3. Ozone and age type effects on yield parameters, g_s and isotopic signals: GY, grain yield (g pot^{-1}); HI, Harvest Index; GNY, grain nitrogen yield (g pot^{-1}); g_s , stomatal conductance ($\text{mmol H}_2\text{O s}^{-1}$); $\delta^{13}\text{C}$, stable carbon isotope composition (‰) of grains; N%, nitrogen concentration (% DW); $\delta^{15}\text{N}$, stable nitrogen isotope composition (‰) of grains. FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L^{-1} of O_3 , NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3 ; Mean values \pm SE. Different letters indicate statistically significant differences in the mean ($p < 0.05$).

		GY	HI	GNY	g_s	$\delta^{13}\text{C}_{\text{grain}}$	N%	$\delta^{15}\text{N}_{\text{grain}}$
Age-Type								
Modern CVs.		30.51 ^b \pm 0.71	0.47 ^b \pm 0.00	0.81 ^b \pm 0.02	0.43 ^b \pm 0.03	−26.55 ^a \pm 0.07	2.67 ^a \pm 0.07	4.30 ^a \pm 0.03
Old CVs.		30.98 ^b \pm 0.53	0.45 ^b \pm 0.01	0.84 ^b \pm 0.02	0.34 ^{ab} \pm 0.03	−25.79 ^b \pm 0.09	2.70 ^{ab} \pm 0.04	4.59 ^a \pm 0.09
Landraces		20.21 ^a \pm 0.75	0.29 ^a \pm 0.01	0.56 ^a \pm 0.02	0.28 ^a \pm 0.02	−24.42 ^c \pm 0.06	2.84 ^b \pm 0.04	5.26 ^b \pm 0.17
<i>p</i> -value		<0.0001	<0.0001	<0.0001	0.002	<0.0001	0.039	<0.0001
Ozone								
FA		28.80 ^b \pm 1.15	0.41 \pm 0.02	0.77 \pm 0.03	0.38 \pm 0.04	−25.88 ^a \pm 0.19	2.70 \pm 0.05	4.66 ^{ab} \pm 0.14
NFA		27.75 ^b \pm 1.24	0.40 \pm 0.02	0.75 \pm 0.03	0.37 \pm 0.03	−25.67 ^{ab} \pm 0.18	2.75 \pm 0.04	4.86 ^b \pm 0.16
NFA+		27.44 ^{ab} \pm 1.15	0.40 \pm 0.02	0.73 \pm 0.03	0.38 \pm 0.04	−25.53 ^{bc} \pm 0.17	2.70 \pm 0.09	4.93 ^b \pm 0.18
NFA++		24.94 ^a \pm 0.90	0.40 \pm 0.01	0.69 \pm 0.03	0.28 \pm 0.02	−25.27 ^c \pm 0.14	2.79 \pm 0.05	4.39 ^a \pm 0.07
<i>p</i> -value		0.003	0.646	0.062	0.120	<0.0001	0.645	0.006
Age \times Ozone								
Cultivars *	FA	32.93 ^b \pm 0.60	0.48 \pm 0.01	0.86 ^b \pm 0.02	0.44 ^b \pm 0.04	−26.56 ^a \pm 0.13	2.63 \pm 0.05	4.35 ^{ab} \pm 0.07
	NFA	32.18 ^b \pm 0.66	0.47 \pm 0.01	0.86 ^b \pm 0.02	0.41 ^{ab} \pm 0.03	−26.28 ^{ab} \pm 0.14	2.69 \pm 0.05	4.48 ^b \pm 0.05
	NFA+	30.55 ^b \pm 0.97	0.46 \pm 0.01	0.79 ^{ab} \pm 0.04	0.41 ^{ab} \pm 0.06	−26.07 ^{bc} \pm 0.15	2.70 \pm 0.05	4.45 ^b \pm 0.06
	NFA++	27.31 ^a \pm 0.80	0.45 \pm 0.01	0.77 ^a \pm 0.02	0.28 ^a \pm 0.03	−25.77 ^c \pm 0.11	2.82 \pm 0.06	4.22 ^a \pm 0.05
<i>p</i> -value (CVs)		<0.0001	0.111	0.005	0.046	<0.0001	0.091	0.008
Landraces	FA	20.52 \pm 1.32	0.29 \pm 0.02	0.57 \pm 0.03	0.241 \pm 0.042	−24.50 \pm 0.10	2.85 \pm 0.09	5.05 \pm 0.31
	NFA	18.89 \pm 1.43	0.27 \pm 0.02	0.53 \pm 0.03	0.284 \pm 0.063	−24.44 \pm 0.12	2.88 \pm 0.08	5.50 \pm 0.38
	NFA+	21.22 \pm 1.87	0.30 \pm 0.02	0.61 \pm 0.04	0.301 \pm 0.058	−24.45 \pm 0.12	2.92 \pm 0.08	5.87 \pm 0.44
	NFA++	20.19 \pm 1.43	0.30 \pm 0.02	0.54 \pm 0.03	0.294 \pm 0.018	−24.29 \pm 0.11	2.73 \pm 0.10	4.74 \pm 0.12
<i>p</i> -value (Land.)		0.748	0.786	0.375	0.821	0.542	0.443	0.089
<i>p</i> -value (Age \times O_3)		0.018	0.261	0.133	0.180	0.179	0.065	0.045

* Cultivars, grouped Modern & Old CVs due to their very similar behavior. Bold highlights statistical significant differences.

These improvements on yield parameters, caused by the varietal selection process, have been accompanied by an increment in the g_s of the CVs. Post-anthesis measurements of g_s were 27% higher in CVs than in landraces.

This same pattern was observed for $\delta^{13}\text{C}$, where landraces showed 2.13 ‰ higher (i.e., less negative) values compared with the modern and old CVs. The breeding process decreased grain N concentration by 6%, while enhancing GNY by 32% for the CVs (modern

and old) vs. landraces. $\delta^{15}\text{N}$ signal decreased by 0.96‰ for the cultivars compared with landraces.

3.3. Ozone Effects

Considering the general response across genotypes, O_3 -exposure affected GY, grain nitrogen yield (GNY) and both isotopic signals (Table 3, Ozone factor). When O_3 increased from FA to NFA++, yield parameters decreased: GY by 13% and GNY by 10% (mean values across the three age-type genotypes), with the effect being highly significant for GY ($p = 0.003$) and a trend near significant ($p = 0.062$) for GNY. Grain size ($p < 0.0001$), biomass ($p < 0.0001$) and Straw Yield SY (also a trend near significant— $p = 0.057$) also decreased by 11%, 9% and 7%, respectively, when FA increased to NFA++ (Appendix A: Table A1). Neither HI nor number of grains per ear were affected by the pollutant.

The observed $\delta^{13}\text{C}$ enrichment induced by the pollutant was small; 0.61‰ in NFA++ compared with FA; however, this response was stable and highly significant ($p < 0.0001$). The significant response of grain $\delta^{15}\text{N}$ to O_3 exposure was not linear: increasing from FA to NFA+ 0.27‰ to then decrease by 0.54‰ from NFA+ to NFA++. Meanwhile, the O_3 -induced reduction observed for g_s was only significant for the CVs.

3.4. Age-Type Genotypes \times Ozone Interactions

Modern and old CVs together responded similarly to O_3 and contrasted with landraces (Table 3, Age \times Ozone). Cultivars subjected to the breeding process, no matter their age, were O_3 -sensitive. Considering yield parameters, the general response was an O_3 -induced yield decrease. The percentage of yield loss for the NFA++ treatment compared with FA across CVs were 17% for GY and 11% for GNY. Meanwhile, grain $\delta^{13}\text{C}$ increased by 0.79‰ (again with a stable and significant response, <0.0001). When only CVs were considered, the O_3 effect on g_s was also significant ($p < 0.05$), inducing a strong (37%) reduction in NFA++ treatment when compared to the FA control. Other parameters affected were grain size and biomass, which decreased by 15% and 11%, respectively. The non-linear O_3 -response pattern observed in the general O_3 -effect on the grain $\delta^{15}\text{N}$ signal was softened but maintained within the CVs group: the pollutant first caused an increase from FA to NFA+ by 0.10‰, to then decrease by 0.23‰ from NFA+ to NFA++. CVs also presented a general N% increasing trend of around 7%. Meanwhile, when landraces were analysed alone, they presented no significant response to the O_3 exposure to any of the parameters assayed.

Nonetheless, despite their O_3 -sensitivity, CVs subjected to genetic selection still presented an overall 34% higher GY when compared to Landraces (mean value across cultivars and O_3 treatments) and maintained this yield advantage under the highest O_3 treatment (26% higher). This breeding improvement under NFA++ exposure also persists when other parameters are considered, such as GNY (30% higher), HI (34% higher) and grain $\delta^{13}\text{C}$ (1.48‰ lower; 6% more negative); or other parameters, such as seed size (25% lower), number of grains per ear (54% higher), SY (41% lower) and total biomass (8% lower) (Table A1). On the contrary, for g_s and nitrogen, the pollutant outweighed the benefits of varietal selection and values between CVs and landraces under NFA++ were similar (without significant differences). For grain $\delta^{15}\text{N}$, the increased discrimination by breeding also remained even when exposed to the highest O_3 treatment, with CVs values 0.52‰ lower than Landraces.

Intra-variability in the O_3 -responses within each of the three age categories of genotypes was lower for the $\delta^{13}\text{C}$ measurements (Figure 3a) than for the g_s measurements (Figure 3b). However, some genotypes differed from the average response of their own group. All of the $\delta^{13}\text{C}$ values for the modern and old CVs, except for Marius, showed a significant response to increasing O_3 levels; however, some exhibited a more sensitive response: Berdún, Califa Sur, Arthur Nick and Ablaca suffered the most intense effect under the NFA++ treatment, with increases ranging between 0.87–1.04‰, while Califa Sur, Yecora and Pane 247 were also significantly affected by the lower O_3 -exposure of the NFA+ treatment (increments from 0.43–0.81‰). This was followed by Pane 247, Yecora and Nogal,

which, although less O₃-sensitive, had a corresponding increased $\delta^{13}\text{C}$ signal ranging from 0.56–0.69‰ under NFA++. In contrast, none of the landraces showed significant changes.

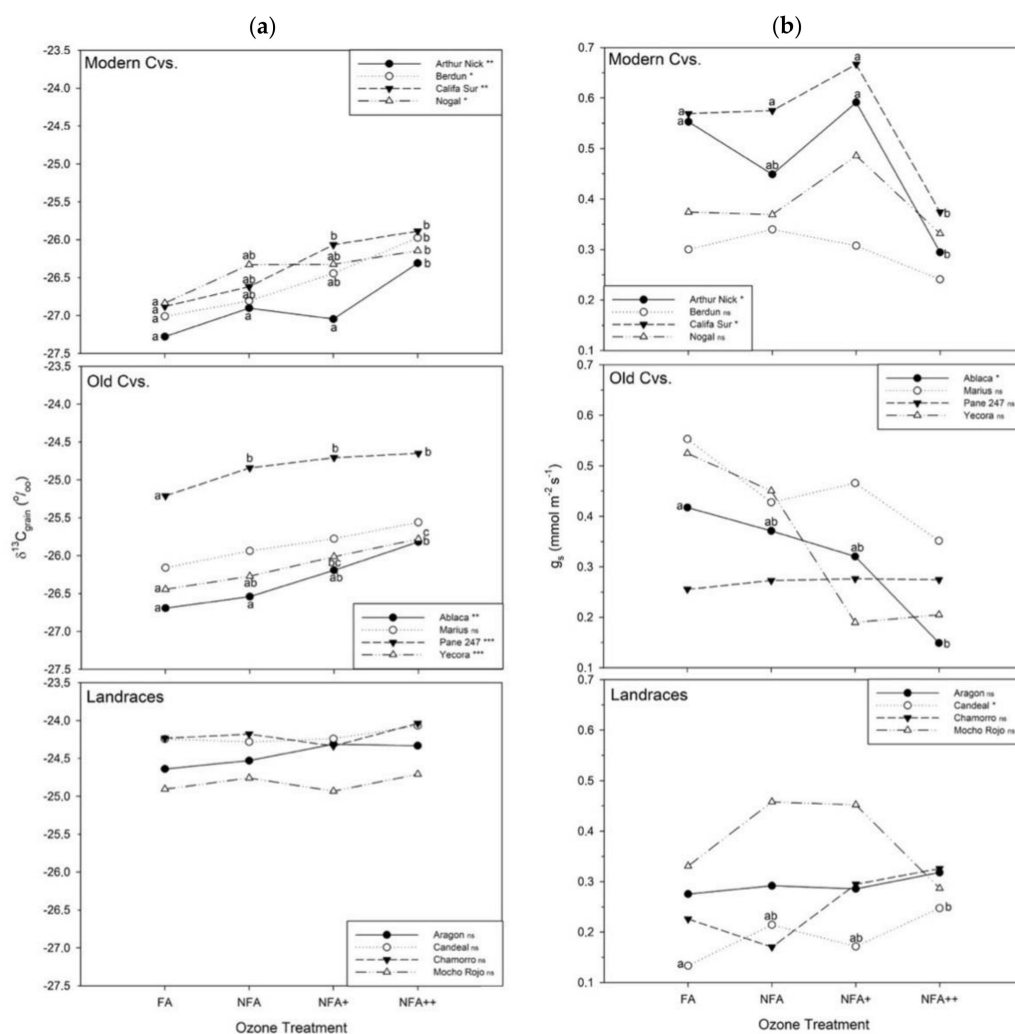


Figure 3. (a) Stable carbon isotope composition ($\delta^{13}\text{C}$; ‰) of mature grains and (b) stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) for the different O₃ treatments: FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L⁻¹ of O₃, NFA++ = non filtered air supplemented with 40 nL L⁻¹ of O₃. Different letters indicate statistically significant differences (** $p < 0.001$, * $p < 0.01$, $p < 0.05$, ns: non-significant) between O₃ levels for each cultivar under Tukey analysis.

When g_s was considered, Califa Sur, Arthur Nick and Ablaca can be considered as the most sensitive; O₃ induced a g_s decrease in the range 34–64% for these CVs (Figure 2). The other $\delta^{13}\text{C}$ -affected CVs presented a non-significant g_s -reduction pattern. A more surprising behaviour was observed in the landrace group for Candéal de Vellisca, whose g_s strongly increased (by 85%) from FA until NFA++.

The same individual analysis per cultivar was done for grain $\delta^{15}\text{N}$ (Figure 4a) and nitrogen concentration (Figure 4b). Individual patterns of the modern and old CVs showed the overall average trend of the CVs group: grain $\delta^{15}\text{N}$ presented an increase in NFA+ and a subsequent drop by NFA++. Marius had the only significant differences in O₃, decreasing from NFA to NFA++ by 0.56‰. Landraces showed a higher variability, and clear amplification of the quadratic response, especially for Chamorro and Candéal de Vellisca; meanwhile, Mocho Rojo presented a decreasing trend. Regarding grain N values (Figure 4b), increases were significant for Arthur Nick and Yecora; Aragon showed an increase until NFA+ but then decreased by NFA++.

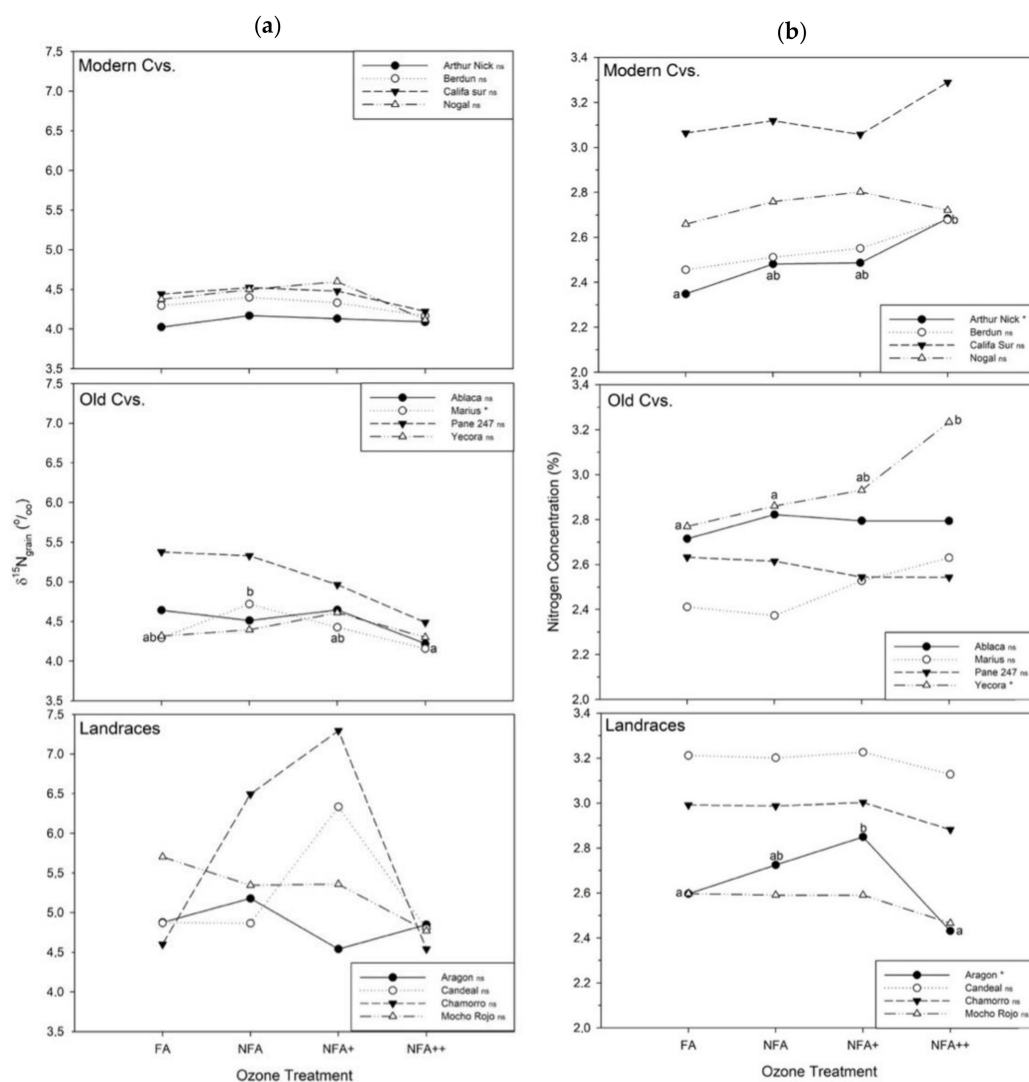


Figure 4. (a) $\delta^{15}\text{N}_{\text{grain}}$ stable nitrogen isotope composition (‰) and (b) nitrogen concentration (N, % DW) for the different O-treatments: FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L⁻¹ of O₃, NFA++ = non filtered air supplemented with 40 nL L⁻¹ of O₃. Different letters indicate statistically significant differences (* $p < 0.05$, ns: non-significant) between O₃ levels for each cultivar under Tukey analysis.

3.5. Yield and Isotopic Signals Correlations

Following the different O₃-sensitivities of the age-type genotypes, grain $\delta^{13}\text{C}$ of modern and old CVs together showed highly significant ($p < 0.001$) negative correlations with GY ($R^2 = 0.60$), GNY ($R^2 = 0.35$), total plant biomass ($R^2 = 0.3$) and significant ($p < 0.05$) negative correlations with HI ($R^2 = 0.18$) and g_s ($R^2 = 0.16$) across O₃ levels (Table 4).

Table 4. Regression analysis for grain $\delta^{13}\text{C}$ (‰) with the assayed yield parameters and g_s : GY, grain yield (g pot^{-1}); Total Biomass (straw yield plus ear and grain, g pot^{-1}); HI, Harvest Index; SY, straw yield (g pot^{-1}); Single sw, single seed weight (mg); GNY, grain N yield (g N pot^{-1}); N%, N concentration (% DW); g_s , stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$); grain $\delta^{15}\text{N}$ (‰). *** Correlation significant at $p \leq 0.001$, ** $p \leq 0.01$ or * $p \leq 0.05$. Pane 247—Old CV, not included.

$\delta^{13}\text{C}$	Modern & Old CVs			Landraces		
	R^2	p -Value	Function	R^2	p -Value	Function
GY	0.5979	***	$y = -6.7319x - 146.98$	0.2705	*	$y = -8.2655x - 181.64$
Total Biomass	0.2899	**	$y = -9.6042x - 173.28$	ns		
HI	0.1769	*	$y = -0.0296x - 0.3083$	0.4434	**	$y = -0.1196x - 2.6314$
SY	ns			0.2876	*	$y = 6.8189x + 215.73$
Single sw	ns			0.2431	*	$y = 2.5208x - 21.953$
GNY	0.3532	***	$y = -0.1221x - 2.3929$	ns		
N%	ns			0.5162	**	$y = 0.6917x + 19.733$
g_s	0.1576	*	$y = -0.1163x - 2.6639$	0.4360	**	$y = -0.2078x - 4.7941$
$\delta^{15}\text{N}$	ns			ns		

Meanwhile, landraces did not present a significant correlation for $\delta^{13}\text{C}$ against total biomass or GNY, but a significant ($p < 0.05$) negative correlation with GY ($R^2 = 0.27$) and highly significant correlation ($p < 0.01$) with g_s ($R^2 = 0.44$). All $\delta^{15}\text{N}$ and g_s correlations for Modern & Old CVs and Landraces were non-significant.

Regarding other traits, the correlation between grain $\delta^{13}\text{C}$ and HI, though still negative, was stronger for landraces ($R^2 = 0.44$) than for modern and old CVs ($p < 0.01$; $R^2 = 0.18$); SY, single seed weight ($p < 0.05$) and grain nitrogen ($p < 0.01$) only showed correlations under the landraces but not for the CVs, with R^2 values of 0.29, 0.24 and 0.52, respectively.

Regression functions for $\delta^{13}\text{C}$ with total biomass, GY, GNY and g_s are shown in Figure 5 specifying the different O_3 treatments with different symbols. It can be observed that the higher O_3 treatments also presented the lowest $\delta^{13}\text{C}$, with some exceptions such as for the tolerant CV Pane 247, which behaves closer to landraces and has not been included in the analysis. No significant correlations were found between grain $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, or between grain $\delta^{15}\text{N}$ or g_s with any of the assayed parameters for both the CVs and landrace groups.

3.6. Principal Component Analysis

The Kaiser–Meyer–Olkin Measure of Sampling Adequacy and Bartlett’s Test of Sphericity deemed the data for each of the three groups of genotypes (general), the modern and old CVs together and the landraces alone as useful for factor analysis (Figure 6a). As harvest index and biomass already correlated strongly with GY and SY, respectively, they were removed to avoid redundancies. Nitrogen concentration was also removed as it had a component matrix under 0.5.

On the general biplot (Figure 6a), both components explained 77.8% of the total variance. Strong differences can be seen between the different age-type genotypes as shown by the separate ellipses. GY, GNY and number of grains per ear were associated positively with g_s , and negatively with grain $\delta^{13}\text{C}$, grain $\delta^{15}\text{N}$, single seed weight and SY. The ellipse embracing the CVs is associated with higher yields, and, to a lower extent, a higher g_s , while the ellipse embracing the landraces is associated with a higher SY and higher grain $\delta^{13}\text{C}$ values. Seed weight does not seem to be generally associated with the other variables, only slightly positively with $\delta^{13}\text{C}$ and SY.

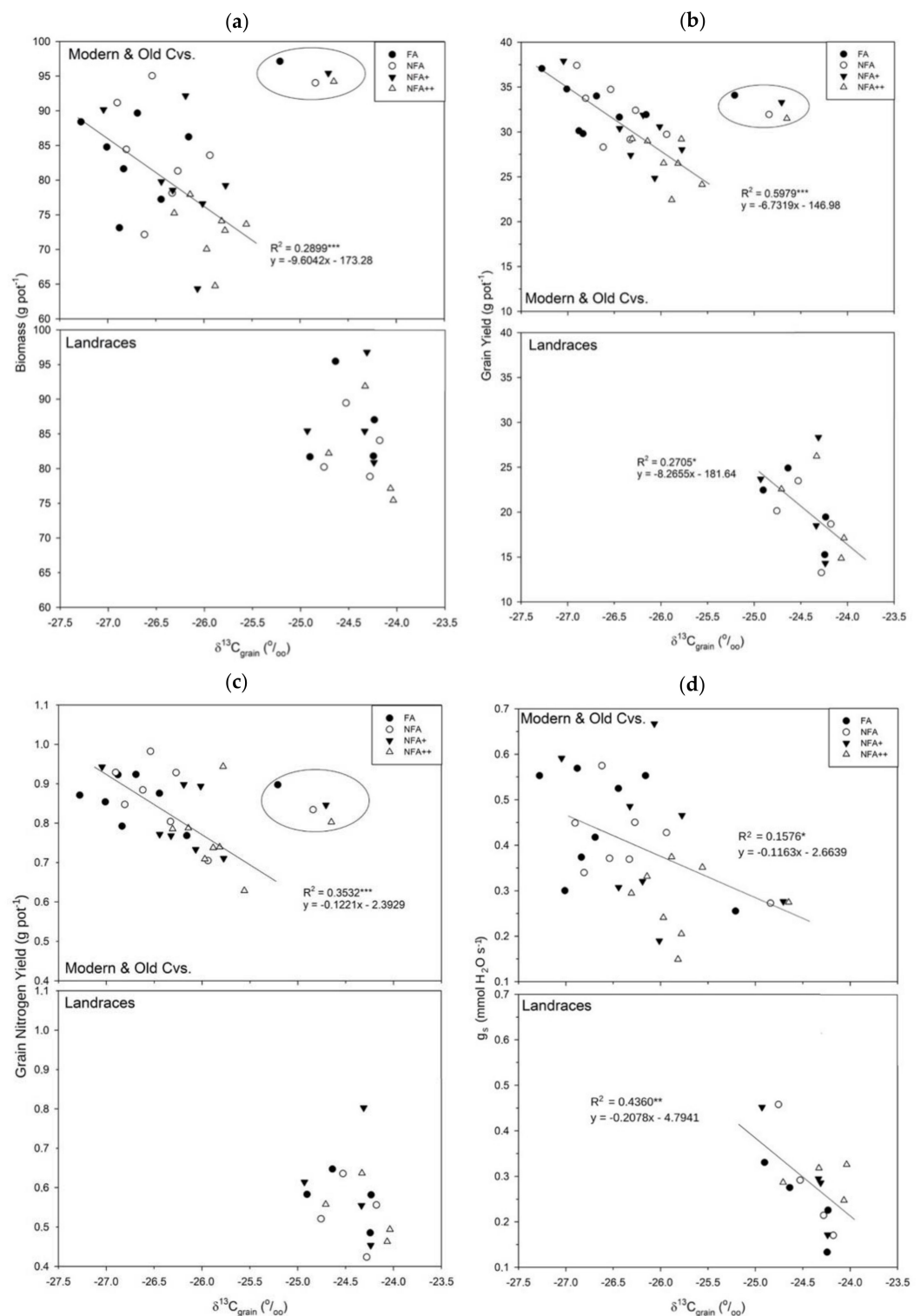


Figure 5. Regression of $\delta^{13}\text{C}$ (‰) with: (a) GY (Grain Yield, g pot⁻¹), (b) GNY (Grain N Yield, g pot⁻¹), (c) total biomass (g pot⁻¹) and (d) g_s (mmol H₂O m⁻² s⁻¹) for the different O_3 -treatments: FA = charcoal filtered air, NFA = non-filtered air, NFA+ = non-filtered air supplemented with 20 nL L⁻¹ of O_3 , NFA++ = non filtered air supplemented with 40 nL L⁻¹ of O_3 . Circle encloses Pane 247—Old CV, not included in correlation analyses. *** significant at $p \leq 0.001$, ** $p \leq 0.01$ or * $p \leq 0.05$.

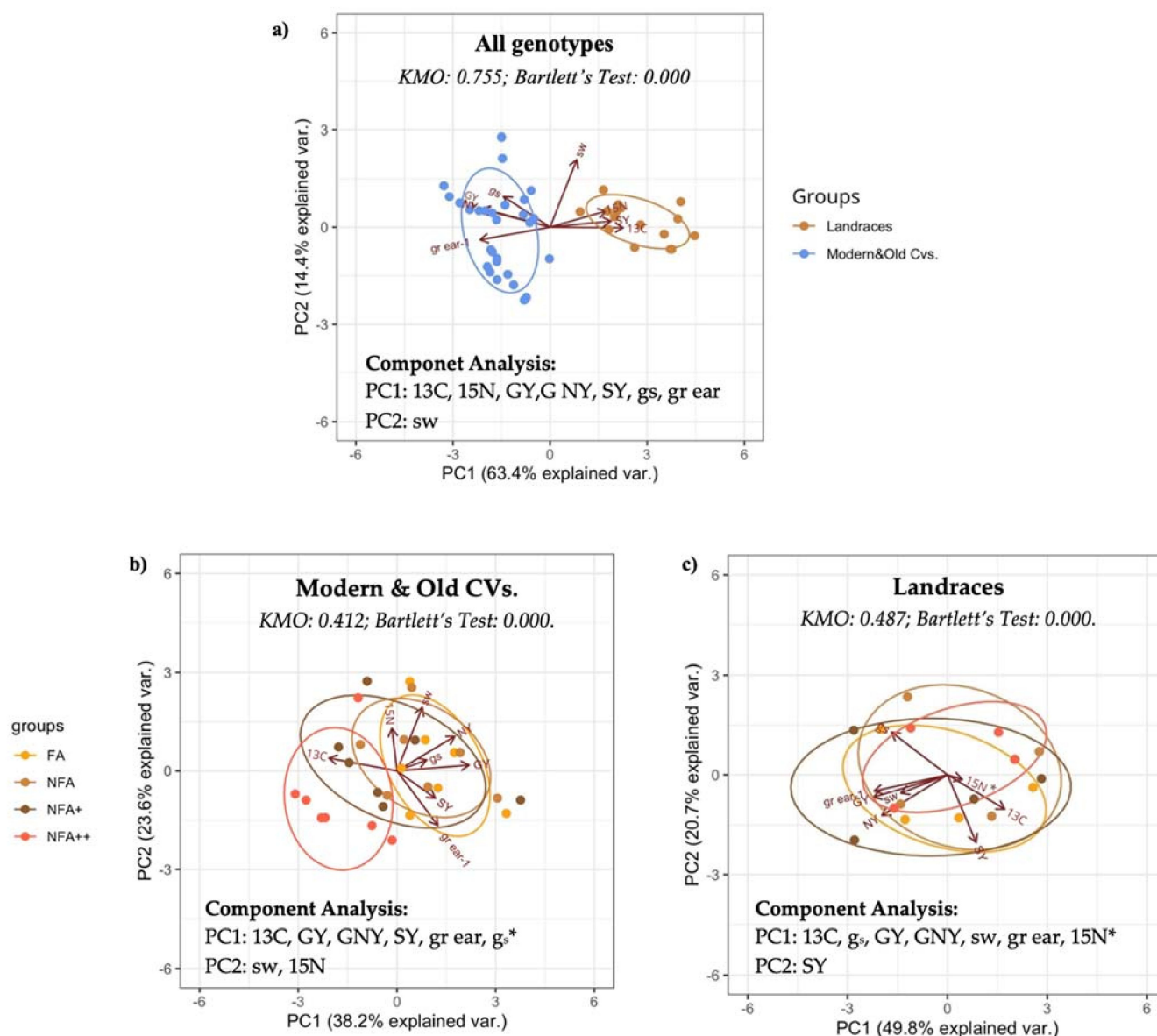


Figure 6. Principal Component Analysis for: (a) each age-type genotype based on standardized values of ^{13}C , $\delta^{13}\text{C}_{\text{grain}}$ (‰); ^{15}N , $\delta^{15}\text{N}_{\text{grain}}$ (‰); GY, grain yield (g pot^{-1}); GNY, grain N yield (g N pot^{-1}); SY, straw yield (g pot^{-1}); sw, single seed weight (mg); gs, stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$); and gr ear $^{-1}$, grains per ear, for eleven (exc. Pane 247) Mediterranean wheat genotypes, (b) Modern & Old CVs and (c) Landraces separated by ozone treatment. * Low Component Matrix value.

When the response to O_3 was considered in the PCA analysis (Figure 6b), modern and old CVs differed from landraces. Landraces showed a great overlap of the ellipses of the different O_3 treatments, while modern and old CVs together show a deviation under the NFA++ treatment. For the modern and old CVs together, both components explained 61.8% of the total variance. PC1 showed negative associations between grain $\delta^{13}\text{C}$, and GY, GNY, SY and number of grains per ear, whereas grain $\delta^{13}\text{C}$ was positively associated with increasing ozone concentrations. In PC2, grain $\delta^{15}\text{N}$ and single seed weight do not seem to be associated with the latter, but correlate positively to each other, though with a short grain $\delta^{15}\text{N}$ vector.

In the landraces, the two components explained 70.5% of the variance. The PC distribution here mirrors the distribution seen in the general analysis, though SY is less

correlated to all the other variables, and seed weight is negatively correlated to the isotopic signals.

For both groups of age-type genotypes, the grain $\delta^{15}\text{N}$ component matrix values were extremely low, lying almost directly on the component 2 axis and following the lack of correlations seen before.

4. Discussion

Spain has the largest surface dedicated to cereal production in the Mediterranean basin [56]. This mainly rainfed, drought-adapted wheat is located in the middle of the Iberian peninsula, where the recorded O_3 values chronically remain high above the protection thresholds for crops and vegetation defined by the European Directive of Air Quality (2008/50/CE) [57]. As hourly O_3 frequently reaches and exceeds 60 nL L^{-1} during early spring [58], which coincides with the non-water limitation period in the area, it allows for maximum physiological activity and gas exchange in the crop and thus, for maximum O_3 absorption [17,26], increasing the O_3 -risk for the wheat despite the characteristic water-deficit later in the season.

During the experiment, the FA O_3 values, in the range $20\text{--}30 \text{ nL L}^{-1}$, well reproduced the natural background levels currently found in the area during the winter and early fall seasons, which are considered as natural O_3 background levels for pre-industrial times [59]; thus, this treatment can be referred to as an adequate control treatment. The NFA treatment represented the daily and seasonal pattern and levels currently registered in the rural areas of the central Iberian Peninsula during spring and summer. It increases from early April until the end of the wheat season, and spans no longer than June, reaching values around 55 nL L^{-1} at the central hours of the day. Similarly, the O_3 levels in the fumigated treatments, NFA and NFA++, reached maximum hourly values of around 90 nL L^{-1} in May, reproducing the maximum hourly values sporadically achieved during the high O_3 episodes in the area [57]. Therefore, the experiment could be considered to adequately represent the poor air quality conditions under which rainfed cereals are growing in the area, and to represent the situation in the Mediterranean basin [60]. This chronic O_3 -pollution is enough to experimentally induce negative effects on crops such as tomato [61], leafy crops [62], watermelon [63] or durum wheat [36]. The latter is more O_3 -tolerant than the spring bread wheat assayed in this study [64]; in fact, bread wheat is currently considered the most economically important sensitive crop and is used as a reference crop for O_3 risk assessment [9]. Air meteorological conditions during the experiment also resembled the characteristic spring-summer conditions of the Mediterranean wheat fields, with temperatures increasing during the season and reaching maximum monthly mean values of around 35°C at the central hours of the day in June, towards the end of the assay, along with the lowest HR value of around 25%.

Results showed strong differences between age-type genotypes, where breeding improved both yield and yield components; for CVs, these effects were strongly related to a decreased carbon isotope composition and an increase in g_s . Landraces and CVs differed significantly; however, old and modern CVs behaved similarly. These results follow previous works performed under field conditions [30], which compared 12 Spanish varieties of soft and durum wheat under different tillage, also considering the age-type factor. Here, landraces had the lowest GY and behaved rather steadily throughout the trial seasons, while CVs presented a higher yield but depended more on the environmental conditions of the year and their independent growth habit. Winter genotypes, such as Marius, presented a higher GY in the humid years, and spring genotypes such as Califa Sur and Yecora performed comparatively better in the dry years. In the present study, no differences were seen due to growth habit since water availability was not a limiting condition in the experimental design and the date of sowing and transplantation was homogenized for all genotypes; however, CVs presented a 34% higher GY than landraces (across all O_3 treatments), which is similar to the 32% observed in Ruiz et al., 2019 [30] across seasons. Nonetheless, differences were seen between genotypes released pre- and

post-green revolution (Table 1), particularly displaying the different behaviour Pane-247 had from the other CVs due to its pre-green revolution release. In this experiment, its very high $\delta^{13}\text{C}$ and low g_s fit much better within the landraces group, which was also released pre-green revolution, causing its removal when all genotypes were joined to analyse the general O_3 response. Actually, strong increases in GY and HI characterized the green revolution; however, further increases in these traits during the following six decades have been comparatively lower, explaining the lack of clear differences between old and modern CVs.

Achieving a higher GY in modern and old CVs was possibly not only due to a higher HI, but also a higher g_s , which favoured photosynthetic activity and caused the more negative $\delta^{13}\text{C}$ values in the newer genotypes [31,42,65]. Therefore, when compared to the landraces, the higher g_s of the post-green revolution cultivars (Table 1) allowed for increased discrimination, favouring the lighter ^{12}C isotope and diminishing the $\delta^{13}\text{C}$ by 2.13‰. A decrease in the $\delta^{13}\text{C}$ (or increase in $\Delta^{13}\text{C}$) has been reported before for Spanish and Italian modern durum wheat cultivars when compared with old cultivars and landraces [42,65], where the difference in $\delta^{13}\text{C}$ between some modern genotypes and landraces from the Iberian Peninsula was around 1.2‰.

However, it should be noted that the higher $\delta^{13}\text{C}$ values seen in the landraces could also be influenced by their longer phenology when compared with the CVs, which exposes them to a more severe terminal (i.e., during grain filling) stress, even under irrigated conditions. The higher g_s observed in the more modern genotypes might seem contradictory for the adaptation process required for the characteristic water limited conditions of Mediterranean rainfed wheat; however, an increase in transpiration would not only aid photosynthesis, but would also limit leaf temperature, which is another important stress that affects plant physiological activity in hot and dry areas [66].

The effect of breeding on increasing $\delta^{15}\text{N}$ and GNY would be associated with the higher assimilation of the more modern varieties, while the observed reduction in grain N% would be related to a grain dilution effect, providing N availability is constant for the plant [28]. In essence, as GY increases with breeding, N% is diluted in the grain when paired with a constant N availability.

The PCA for the combined modern plus old CVs group and the landraces group (Figure 6a) reinforced the aforementioned two very different behaviours. The PCA pattern of the analysed parameters, except for seed weight, which did not correlate well with the others, showed the expected behaviours for the two age-groups, where the yield increase (GY and GNY) linked to breeding selection was positively associated with g_s , and negatively associated with the isotopic $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signals and SY. This behaviour is opposite to that of the landraces.

In terms of O_3 -response, the effect was age-type dependent; while modern and old CVs presented an O_3 -induced reduction in yield parameters and g_s , as well as an alteration of both isotopic signals (less negative $\delta^{13}\text{C}$ and lower $\delta^{15}\text{N}$), landraces were not affected by O_3 exposure, even under the highest levels of the NFA++ treatment. The similarity in the O_3 response of the post-green revolution CVs (modern and old CVs without Pane 247 as shown in Table 1) was striking and allowed them to be analysed jointly. Exposure to O_3 induced high yield losses, 17% and 11 % for GY and GNY, respectively, as an average value across CVs in the NFA++ treatment. A meta-analysis derived by Feng et al., 2008 [67], considering more than 50 studies, obtained mean GY losses of 29% in modern wheat varieties for O_3 -exposures based on Accumulated Ozone Exposure (AOT) functions that were slightly higher than the present NFA++ treatment. Meanwhile, in a review for Europe-level O_3 effects, similar O_3 exposures between 80–100 ppb produced yield reductions that rounded 30% [68]. Compared with these results, the Spanish CVs seem to lie within the O_3 -sensitivity range observed for modern wheat CVs from other agronomic regions and for Central and North European levels, which do not have the characteristic water limitation of the Mediterranean region.

The O₃ effects on yield parameters of the sensitive CVs paralleled those of the g_s reduction. The greater sensitivity of modern CVs, compared to traditional genotypes not subjected to a programmed selection process, has been observed in previous works on wheat [28,32]. All of them showed smaller O₃-effects on the lower yielding old cultivars, and associated breeding selection for higher yield with an increased gas exchange via higher g_s, which led to an unintentional greater O₃ absorption and damage. Other studies have defined some of the O₃-damage mechanisms that affect modern varieties, which relate the O₃ induced lower photosynthetic rates to non-stomatal factors, such as a decreased carboxylation or electronic transport and lower capability of the antioxidative system [69].

Previous studies have concluded that, for wheat, the most O₃-sensitive period is anthesis, as O₃-effects on previous stages were not statistically significant [37]. Therefore, the present experiment was designed to start fumigation after spike emergence, close to anthesis. Accordingly, the overall negative O₃-effects were detected more in grain parameters than in biomass parameters, which follow the observed decrease in HI induced by the pollutant. The GY reduction was caused mainly by smaller grains while maintaining the number of grains per ear, also following results found in previous studies [68].

While this experiment followed many of the yield related O₃-effects seen in previous studies, few studies have delved into the effects O₃ has on the isotopic signature of wheat [25,40], and none have sought this for Mediterranean wheat. In experimental assays, gas exchange and carbon fixation factors affected by O₃ are often measured sporadically, and the results are extrapolated to the entire exposure period; even though these parameters usually present daily and seasonal patterns depending on the plant development stage [28], which might lead to a misinterpretation of the O₃ response. As $\delta^{13}\text{C}$ in plant dry matter depends on both stomata and Rubisco carboxylation [46], if the stomata close or their conductance decreases due to any stress, such as high O₃, stomatal discrimination decreases, restricting the CO₂ supply to the carboxylation sites and overall increasing $\delta^{13}\text{C}$ (or decreasing $\Delta^{13}\text{C}$) [39,40]. The integrated effect on the physiological activity of the plant throughout its lifespan, for whichever metabolic route the pollutant has affected, can be assessed by considering the isotopic parameters [43].

Results of the present work confirm the starting hypothesis that the $\delta^{13}\text{C}$ of mature grains becomes less negative (increases) under O₃ stress for O₃-sensitive CVs, due to the g_s reduction induced by the pollutant, which both decreases the capacity of the plant for carbon discrimination and negatively affects photosynthesis, decreasing GY. On the contrary, the landraces O₃-tolerance correlates with the non-effect seen in $\delta^{13}\text{C}$ of mature grains, along with a decrease in g_s and yield parameters. This also follows the postulate that, under stress conditions, cultivars use their current photosynthetic activities to produce carbohydrates for grain filling, while landraces rely more on storing carbohydrates in the stem to then redistribute them to the grain [28,29]. This could suggest that if the landraces had been exposed to ozone earlier, when they were storing carbohydrates in the stem, they could have shown a more sensitive behavior in their GY, which could be analyzed with an experiment with chronic ozone exposure beginning before anthesis.

Both the regression functions and the PCA analysis pointed to these results. It should be noted that a negative correlation between $\delta^{13}\text{C}$ of mature grains and g_s was observed within CVs and landraces; however, for the latter there was no direct relationship with the pollutant. The observed results of the PCA for each of the O₃ treatments (Figure 6b) reiterate the commented O₃-tolerance of the landraces, and the sensitivity of the modern and old CVs, particularly under the NFA++ treatment related to the decreased g_s, and hence, in ^{13}C discrimination (and thus higher $\delta^{13}\text{C}$), and lower GY, GNY, SY and number of grains per ear. Meanwhile, for both CVs and landraces, this effect is not shown in single seed weight, nor grain $\delta^{15}\text{N}$, most possibly due to its more complex isotopic fractionation processes [47].

The general response pattern of the grain N concentration, which increases under increasing O₃ levels, was more evident under the sensitive CVs, presenting a 7% increase due to the pollutant. This aligned with previous studies that analysed wheat grown under

O₃ stress [28,70], and has also been seen with drought [71]. As senescence is accelerated by O₃-stress, it would favour N translocation to the reproductive parts, enriching the grain more with protein than starch [72]; moreover, as the individual grain becomes smaller, it further increases the concentration [19]; however, when coupled with a decreasing GY, it actually leads to an overall decrease in GNY.

In general, previous studies on wheat indicate that when there is a positive O₃-effect on N grain concentration, there is an approximately equally negative effect on the yield [73]. Therefore, even though grain quality improves, the amount of protein accumulated per unit area, related to GNY, decreases, which can lead to food security and economic problems. In the present study, the effect on GNY was softer than the one observed in yield loss, with a GNY reduction of 11% compared to the 17% found for GY. This could be because O₃ exposure began close to anthesis, and even though this period is the most O₃-sensitive for wheat [37], it does not match the main period for N absorption, which usually decreases greatly after anthesis and during grain filling [50]. At anthesis, wheat focuses more on N redistribution from the vegetative tissues (e.g., flag leaf) for grain filling [47]. Therefore, a reason for a lack of stronger differences regarding the N-related parameters could be because N uptake from the soil had greatly decreased before the O₃ treatment began. Nonetheless, the landraces are characterized by a later phenology, in the sense that heading and anthesis occur later than in CVs; however, in the present study, all the genotypes were homogenized for growing so differences caused by their growth habit would not apply here.

As landraces rely more on ears, which are generally less affected by stress, and on protein degradation from other plant organs during grain filling stages [49,74], the O₃-effect on assimilation and redistribution could be particularly detrimental to the sensitive CVs when under stress; since they rely more on the flag leaf for N translocation for grain filling which senescences faster under O₃ exposure. At the same time, as active nutrient uptake by the roots occurs at early spring, future experiments designed to begin O₃ fumigation at the pre-anthesis stage are needed to take O₃ effects on N into consideration, especially for rainfed wheat.

Concerning the $\delta^{15}\text{N}$ of mature grains, as the plants were grown in pots with artificial substrate and had the same N fertilizer source (with a given $\delta^{15}\text{N}$), the starting pattern of assimilation and further fractionation is expected to be very similar for all the plants. $\delta^{15}\text{N}$ values are largely driven by nitrogen metabolism, including nitrogen assimilation, recycling and redistribution [38,47–49]. When N moves from one organ to another, the $\delta^{15}\text{N}$ signal in the organ increases as it builds up moving upwards towards the grain [47]. Therefore, alterations in plant metabolism and growth can modify $\delta^{15}\text{N}$. However, in our study, the assimilated N was accumulated in the flag leaf before the O₃ fumigation treatment began [49]. Due to the low levels of N absorption after anthesis [73], this suggests that only processes related to the N translocation from the leaves to the grains during grain filling were responsible for the effect of O₃ on $\delta^{15}\text{N}$, relating the O₃ response of the N parameters, such as $\delta^{15}\text{N}$, more with N redistribution.

The general response of $\delta^{15}\text{N}$ of the grain to the pollutant followed a non-linear pattern, upon which it increased from the control to the NFA+ treatment, to then being decreased by NFA++. Here, the effect of O₃ on $\delta^{15}\text{N}$ of grains was significantly driven by the CVs as opposed to the landraces and could again be related to the higher O₃-sensitivity of the CVs as well as on their dependence on N redistribution from organs that are highly affected by O₃, such as the flag leaf. Meanwhile, as mentioned, N redistribution from the ears, which is more associated to landraces, works well both with and without stress [49], giving landraces an advantage in redistribution under O₃-stress. The negative correlation between $\delta^{15}\text{N}$ and GY has been observed before [42], although it was not significant in the present assay. An alternative explanation for the decrease in $\delta^{15}\text{N}$ of the CVs as a response to the more severe O₃ treatment might be related to the decrease in g_s , which would limit volatilization of the lighter ¹⁴N isotope with relation to the heavier ¹⁵N [47,49,75], in turn leading to a lower $\delta^{15}\text{N}$ of mature grains.

It should also be noted that the landraces displayed a large heterogeneity in the $\delta^{15}\text{N}$ signal compared to the CVs, which could be due to their higher genetic variability [76] coupled with the aforementioned N redistribution from the ears and protein degradation of other plant parts [49]. The importance of the N redistribution mechanisms in the grain N should be considered for future plant breeding programs.

5. Conclusions

Modern and old CVs behaved very similarly under O_3 exposure, showing a high O_3 -sensitivity based on the significant impacts seen on yield parameters, such as GY or GNY, and biomass, and, accordingly, on g_s and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. On the contrary, landraces showed a stable and tolerant behaviour under the increasing O_3 exposures.

A different behavior was found between genotypes depending on their release date, where pre-green revolution genotypes presented very high $\delta^{13}\text{C}$ and low g_s .

$\delta^{13}\text{C}$ grain correlated strongly with both stomatal conductance and yield factors such as GY, GNY or total biomass; therefore, $\delta^{13}\text{C}_{\text{grain}}$, which can conglomerate all the intrinsic mechanisms by which the plant reacts to chronic O_3 exposure, and which hinder its yield, could be used as a good indicator of the cumulative effects of O_3 at the end of the wheat plant lifespan. Moreover, $\delta^{13}\text{C}$ values presented a more homogeneous response within each age-type group than other physiological response parameters such as g_s , backing its potential use as an indicator for accumulated O_3 effects.

Since the O_3 exposure period started post-anthesis, $\delta^{15}\text{N}_{\text{grain}}$ response was more indicative of alterations caused by the pollutant on N redistribution and N volatilization, and requires a more integrated future analysis due to its dependence on other N-related factors, such as absorption, metabolism and redistribution.

In the present study, the benefits of modern breeding through varietal selection mostly counterbalanced the negative O_3 effects on the modern and old CVs; however, the strong differences found in O_3 -sensitivity between CVs and landraces indicate that there is still a broad genetic base for improving O_3 -tolerance to enhance productivity and profits in Mediterranean agriculture, and to better adapt Mediterranean wheat to global change factors such as chronic high O_3 levels.

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Appendix A

Table A1. Ozone and age type effects on yield parameters: SY, straw yield (g pot^{−1}); Single sw., single seed weight (mg); # grains ear^{−1}, number of grains per ear per pot; Biomass, added value of the straw yield and the ears with grains (g pot^{−1}). FA= charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L^{−1} of O₃, NFA++ = non filtered air supplemented with 40 nL L^{−1} of O₃; Mean values ± SE. Different letters indicate statistically significant differences ($p < 0.05$).

		SY (g)	Single sw. (mg)	# Grains Ear ^{−1}	Biomass (g)
Age Type					
Modern CVs.		33.61 ^a ± 0.66	31.57 ^a ± 0.66	44.18 ^c ± 1.07	78.42 ^a ± 1.24
Old CVs.		37.77 ^b ± 1.08	38.08 ^b ± 0.90	36.66 ^b ± 1.08	85.16 ^b ± 1.40
Landraces		49.22 ^c ± 0.63	39.61 ^b ± 0.33	18.61 ^a ± 0.93	84.61 ^b ± 1.05
<i>p</i> -value		<0.0001	<0.0001	<0.0001	<0.0001
Ozone					
FA		40.94 ± 1.49	37.85 ^b ± 0.93	33.55 ± 2.18	85.36 ^b ± 1.31
NFA		40.97 ± 1.34	37.18 ^b ± 0.83	32.98 ± 2.19	84.38 ^b ± 1.27
NFA+		40.96 ± 1.56	36.95 ^b ± 0.90	33.25 ± 2.14	83.74 ^b ± 1.61
NFA++		37.93 ± 1.38	33.69 ^a ± 1.06	32.81 ± 2.15	77.44 ^a ± 1.52
<i>p</i> -value		0.057	<0.0001	0.975	<0.0001
Age × Ozone					
Cultivars *	FA	36.18 ± 1.26	36.71 ^b ± 1.33	41.18 ± 1.64	84.79 ^b ± 1.67
	NFA	36.80 ± 1.22	36.02 ^b ± 1.13	40.76 ± 1.57	84.99 ^b ± 1.66
	NFA+	36.41 ± 1.58	35.47 ^{ab} ± 1.22	39.76 ± 1.85	82.04 ^{ab} ± 2.14
	NFA++	33.38 ± 1.19	31.10 ^a ± 1.25	39.96 ± 1.79	75.34 ^a ± 1.89
<i>p</i> -value (CVs)		0.248	0.008	0.929	0.001
Landraces	FA	50.45 ± 1.51	40.13 ± 0.48	18.31 ± 1.56	86.50 ± 2.13
	NFA	49.32 ± 1.17	39.52 ± 0.73	17.41 ± 1.57	83.15 ± 1.93
	NFA+	50.06 ± 1.25	39.90 ± 0.61	20.23 ± 2.56	87.13 ± 1.98
	NFA++	47.04 ± 0.94	38.87 ± 0.79	18.51 ± 1.75	81.66 ± 2.17
<i>p</i> -value (Land.)		0.219	0.558	0.764	0.193
<i>p</i> -value (Age × O ₃)		0.946	0.289	0.767	0.218

* Cultivars, grouped Modern & Old CVs due to their very similar behavior. Bold highlights statistical significant differences.

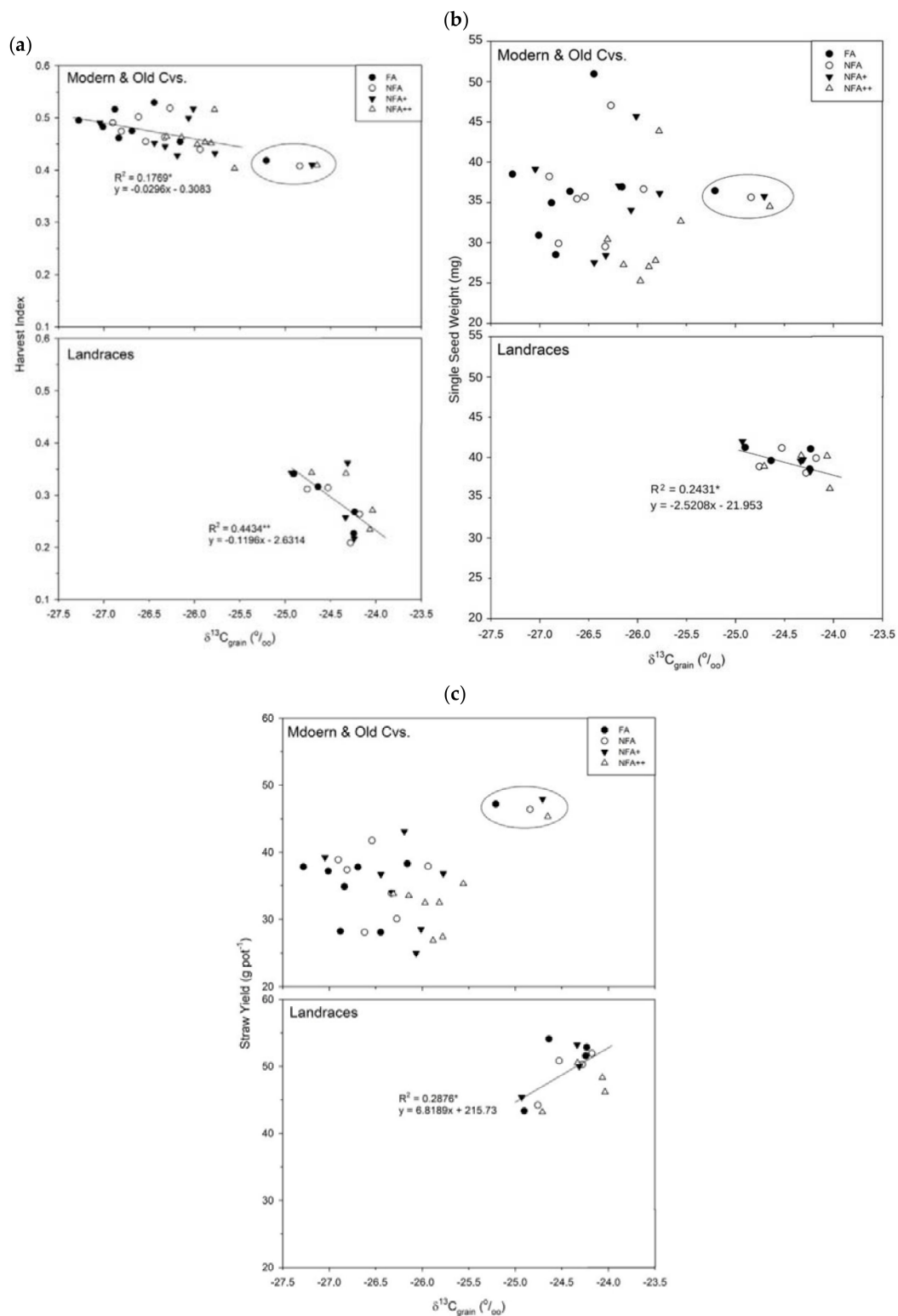


Figure A1. Regression functions of $\delta^{13}\text{C}$ (stable C isotope composition, ‰) with: **(a)** Harvest Index, **(b)** Single seed weight (mg) and **(c)** Straw yield (g pot^{-1}) for the different O_3 -treatments: FA= charcoal filtered air, NFA = non-filtered air, NFA+ = non-filtered air supplemented with 20 nL L^{-1} of O_3 , NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3 . Circle encloses Pane 247—Old CV, not considered for correlation analyses. Correlation is significant at $** p \leq 0.01$ or $* p \leq 0.05$.

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